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Adapter for coupling a substance to be coupled to a cell surface

The invention relates to adapters for coupling a substance to be coupled to a cell surface. The invention renders it possible also to couple to the surface of a cell those substances which normally, i.e. without the adapter, require a particular surface structure on the cell surface (usually a receptor) for coupling. The invention relates in particular to adapters for coupling an adenoviral fibre knob protein to a cell surface, as well as nucleic acids which code for these adapters, viruses and methods for their use. The invention also relates to substances and methods for mediation and/or improvement of the coupling of substances, such as adenovirus particles, to a cell surface which contains little coxsackie adenovirus receptor or none at all.

Transgenic adenoviruses are often used for transfer of heterologous nucleic acids into eukaryotic cells, in particular mammalian cells. These carry on the surface of the virus particle a protein, called fibre knob, which can be recognized and bound by a receptor protein arranged on the surface of the cell to be treated (target cell) and called

coxsackie adenovirus receptor (CAR; Bergelson, J.M. et al., Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5; Science 275 (1997), 1320-1323). Binding of the fibre knob protein to CAR is the rate-determining step of the adenoviral infection process. It is assumed that after binding of the coxsackie adenovirus receptor to the fibre knob protein and the associated coupling of the virus particle to the cell surface, a further adenovirus envelope protein, the penton protein, binds to a constituent of the cell surface, namely to one or more integrins, as a result of which endocytosis of the virus particle into the target cell is induced.

The dependency of adenoviruses on the presence of the coxsackie adenovirus receptor on the surface of the target cell limits the usability of adenoviruses for transfer of heterologous nucleic acids into eukaryotic cells (Kim, M., et al., The therapeutic efficacy of adenoviral vectors for cancer gene therapy is limited by a low level of primary adenovirus receptors on tumor cells; Eur. J. Cancer 38 (2002), 1917-1926). Furthermore, it has emerged that expression of CAR depends on the degree of differentiation of a cell (Walters, R., et al., Adenovirus fiber disrupts CAR mediated intercellular adhesion allowing virus escape; Cell 110 (2002), 789-799). The transfer rate of heterologous nucleic acids into tumour cells hitherto achievable with adenoviruses is accordingly unsatisfactorily low.

Attempts have therefore been made to modify the section of the fibre knob protein which is decisive for CAR recognition in order to render it possible for the viruses to bind to other constituents of the cell surface than merely the coxsackie adenovirus receptor, and thus to modify the tropism of the viruses. However, this set-up is very expensive, since it necessitates a modification of the virus genome. Furthermore, the capacity of the fibre knob protein for genetic modifications is very limited (Suzuki, K., et al, A conditionally replicative adenovirus with enhanced infectivity shows improved oncolytic activity, Clin. Cancer Res. 7 (2001), 120-126).

Attempts have likewise been made to prepare fusion proteins which comprise the extracellular domain of the coxsackie adenovirus receptor which is responsible for the fibre knob protein recognition and a ligand section for recognition by a receptor expressed on the surface of the target cell (target receptor) (Pereboev, A.V., et al.,

Coxsackievirus-adenovirus receptor genetically fused to anti-human CD40 scFv enhances adenoviral transduction of dendritic cells, Gene Ther. 9 (2002), 1189-1193). This set-up enable cells which express no coxsackie adenovirus receptor or express it to only a small extent but express at least one other receptor to be infected with adenoviruses. Disadvantages are, however, that with this set-up a new fusion protein must be produced for each target receptor, and that the infection remains limited to those target cells which express the target receptor (Curiel, D.T., Considerations and challenges for the achievement of targeted gene delivery, Gene Ther. 6 (1999), 1497-1498). This limits the usability of the fusion proteins in particular to infection and treatment of tumours. In the course of their development, tumour cells become increasingly genetically unstable and change the pattern of the receptors expressed by them; each tumour nodule accordingly has its own spectrum of cell surface receptors. For treatment of tumours, it would therefore be necessary first to determine the receptors expressed by all the tumour cells, in order subsequently to prepare corresponding fusion proteins.

A method for increasing an adenovirus-mediated gene transfer is known from the publication by Gratton et al., Nature Medicine 2003, 357-362. In this, adenoviruses are preincubated with high concentrations (0.05 to 5 mM) of the third α-helix of the Antp homoeodomain and then added in dilute form to the cells to be transduced. An improved transduction of CAR-free COS-7 cells was observed during this treatment. A significant improvement in the transduction performance was observed neither with preincubation of the COS-7 cells with the third α-helix of the Antp homoeodomain and subsequent addition of the adenovirus nor with preincubation of the COS-7 cells with adenovirus and subsequent addition of the third α-helix of the Antp homoeodomain. A disadvantage of the treatment method described by Gratton et al. is that the adenoviruses envisaged for the transduction must be pretreated with very high concentrations of the third α-helix of the Antp homoeodomain. As a result, no permanently improved transduction performance of the adenoviruses used is achieved, and instead the transduction performance only of the pretreated viruses is improved. It is a disadvantage for gene therapy purposes in particular that cells which have been infected with an adenovirus pretreated by the method of Gratton et al. can produce no adenoviruses which have a comparably improved transduction

performance. For this, the cells would also have to be able to express the third α-helix of the Antp homoeodomain in the high concentrations described by Gratton et al., but this cannot be achieved physiologically.

The object of the present invention was therefore to remedy the disadvantages described above and to provide means and methods to extend in the simplest possible manner the spectrum of cells which can be infected by adenoviruses (target cells). Infection at an acceptable or good rate of those cells which express no coxsackie adenovirus receptor on their surface, or express one to only a small extent, should advantageously also be rendered possible. The means and methods should be usable not only in the context of research, but furthermore preferably also therapeutically, in particular in gene therapy, such as, for example, for tumour treatment, and/or non-therapeutically, in particular for infection of cell cultures.

There is moreover also generally a need to be able to couple a substance to the surface of a target cell in the simplest possible manner, whereby it should be possible to choose the target cell from the widest possible spectrum of various cell types.

According to the invention, an adapter for coupling a substance to be coupled to a cell surface is provided, comprising

a) a section for recognition of and binding to the substance to be coupled, and

b) a recombinant section for arranging the adapter on the cell surface having an affinity for one or more negatively charged cell surface structures.

An adapter according to the invention can simultaneously recognize the substance to be coupled and bind to it, and optionally be arranged on the cell surface by mediation of further structures and elements of the cell surface, in particular by mediation of widespread receptors. Production, for each cell type of a selected target cell, of a fusion protein matched to the specific receptor expression pattern thereof can thus be largely avoided in an advantageous manner.

In the context of this invention, "recognition" is understood as meaning the ability of the adapter to bind with an on average higher affinity to the substance to be coupled (in particular to the adenoviral fibre knob protein) than to another random protein. The recognition and binding of the adapter according to the invention to the substance to be coupled can in general be regarded as ligand-receptor interaction, but is not limited to such a mechanism.

Section b) of the adapter according to the invention renders possible the arrangement of the adapter on the surface of the target cell. In this context, arrangement of the adapter on the surface of the target cell is understood as meaning connecting of the outer cell surface to the adapter, the connection not being merely random and fleeting. Instead, the adapter according to the invention has a high affinity for the cell surface, in particular for some cell surface elements on the outside of the cell surface, such as proteins and carbohydrates. The adapter also remains arranged outside on the cell surface for a period of time, and is not immediately internalized into the target cell, and/or the adapter remains available in the extracellular space for recognition and binding of the substance to be coupled. Preferably, the adapter has a long half-life of medium availability, so that the internalization rate is not more than 5 pmol of adapter/h, preferably not more than 3 pmol of adapter/h (under the following assay conditions: 1 x 10^6 target cells in 3 ml DMEM/10% FCS).

The arrangement on the cell surface is preferably effected by coupling of the adapter according to the invention to cell surface elements which are widespread or ubiquitous in various cell types. In particular, the cell surface elements can be receptors. According to first studies, the negatively charged elements of the cell surface, to which an adapter according to the invention presumably binds, have no signal transmission function.

In preferred embodiments, section b) has an overall basic character and/or is preferably positively charged. Peptides having a basic character and positively charged peptides are particularly suitable for permanent arrangement on cell membranes, without relying on cell surface elements specific to the cell type for this

arrangement. In this context, positively charged peptides can add on readily to negatively charged structures of the cell surface.

Sections a) and b) of the adapter are advantageously covalently bonded to one another, optionally via a further connecting section.

In preferred embodiments, the adapter is advantageously equipped by its section a) for recognition and binding to a virus or another, preferably virus-like substance, in particular to an adenovirus, but also with the aid of other, in each case suitable sections a) to a cytomegalovirus (CMV), herpes simplex virus (HSV), reovirus and vesicular stomatitis virus (VSV). The adapter is preferably equipped, in particular by its section a), for recognition and binding of a surface structure (linker) specific for the particular substance to be coupled (in particular for the particular virus), and thus renders it possible for the virus or another substance to be coupled comprising the particular surface structure (linker) to be arranged selectively on the cell surface of a target cell. The term "specific" in this connection means that the linker makes the substance to be coupled at least so readily distinguishable from other substance which conventionally likewise occur on the target cell on administration of the substance to be coupled that under these conditions the adapter recognizes and binds chiefly the substance to be coupled and not chiefly one of the other substances.

In particularly preferred embodiments, section a) comprises a section for recognition of and binding to the adenoviral fibre knob protein. A corresponding adapter can simultaneously couple to a conventional adenoviral fibre knob protein and be arranged on the cell surface by mediation of further structures and elements of the cell surface, in particular by mediation of widespread receptors, or without mediation of further elements of the cell surface. Having to genetically modify the adenoviruses themselves to extend the spectrum of cells which can be infected by adenoviruses can thus be avoided in an advantageous manner; instead, conventional adenoviruses can also be used for infecting target cells which were hitherto infectable or only poorly infectable. Moreover, by providing the (recombinant) adapter according to the invention, the invention also bypasses, for many cell types, the need to have to produce for each cell

type to be infected a fusion protein adapted to the native receptor expression pattern thereof.

It is particularly preferable if section a) comprises a part of the extracellular domain of coxsackie adenovirus receptor (CAR) which is functional for recognition of the fibre knob protein, or a part which has the equivalent function thereto.

Section a) of such an adapter contains a part of the extracellular domain of the coxsackie adenovirus receptor (that is to say optionally also the complete extracellular domain) or a part of equivalent function thereto. In this context, a section a) which can recognize an adenoviral fibre knob protein, bind thereto and thus couple the adapter according to the invention to the fibre knob protein is of equivalent function. To select section a), the person skilled in the art can make use of the known literature relating to the construction and function of the coxsackie adenovirus receptor and correspondingly easily find an amino acid sequence which is suitable for coupling the adapter to a fibre knob protein. Amino acids 1-235 of the coxsackie adenovirus receptor (Pubmed Accession No.: NM 001338, all the following Accession Numbers likewise from Pubmed) are particularly preferred as section a).

Furthermore, in addition to or instead of a part of the extracellular domain of the coxsackie adenovirus receptor, the person skilled in the art can envisage in section a) a part which indeed still renders possible coupling of the adapter according to the invention to the fibre knob protein, but is not contained as such in the extracellular domain of the coxsackie adenovirus receptor. In particular, the person skilled in the art can omit, add and/or replace by other amino acids individual amino acids of the extracellular domain of the coxsackie adenovirus receptor, as long as the function of section a) is retained, namely rendering possible coupling of the adapter according to the invention to a fibre knob protein.

With the adapter according to the invention it is thus possible for the first time, without detailed previous knowledge of the receptors expressed by the target cell, to arrange an adapter having a high affinity for an adenoviral fibre knob protein on the surface of a target cell and thus to render this receptive for coupling of an adenovirus particle to

the target cell. The adapter according to the invention renders it possible in an advantageous manner to infect with adenoviruses a high proportion of epithelial cells, fibroblasts, neuronal cells, dendritic cells, osteoblasts and myocytes as well as degenerated cells originating from these cell types.

An adapter in which section b) is chosen from:

- a peptide 6 or more amino acids long, at least half of which contain guanidino and/or amidino side chains,
- an oligopeptide having at least 6 arginine side chains,
- a peptide of the amino acid sequence corresponding to the basic region of the HIV tat protein,
- the third helix of a homoeobox protein, and
- a section of protein VP22 of the herpes simplex virus which mediates anchoring to a cell surface,

is preferred.

In this context, those adapters in which section b) is chosen from:

- a peptide of amino acid sequence corresponding to the basic region of the HIV tat protein,
- a section of protein VP22 of the herpes simplex virus which mediates anchoring to a cell surface,

are particularly preferred.

These adapters have proved, significantly better than adapters with the remaining sections b) mentioned, to be particularly suitable in a large number of cell types for coupling a substance to be coupled, in particular for coupling an adenovirus (in this case section a) of the adapter preferably comprises a part of the extracellular domain of the coxsackie adenovirus receptor (CAR) which is functional for recognition of the fibre knob protein, or a part of equivalent function thereto).

It has indeed already been investigated beforehand whether peptides of the preferred characterizing type are suitable for arrangement on the surface of eukaryotic cells. However, it is not been disclosed to provide these peptides with a section for recognition and binding of a substance to be coupled, in particular the adenoviral fibre knob protein. Furthermore, it has now been found that the said peptides not only are suitable for transporting macromolecules into cells, but also (in combination with a section a) which recognizes and binds an adenovirus particle) can effect an addition of the adapter according to the invention on to the cell surface of a target cell which is sufficiently permanent for the purpose of infection by adenoviruses.

US 6,495,663 thus discloses a transport polymer of 6 to 25 subunits, at least 50 % of which contain a guanidino or amidino side chain. The transport polymer is suitable for transporting an agent appended thereto through a biological membrane at a rate which is higher than that of the agent as such. To develop the adapter according to the invention, the person skilled in the art will therefore expediently orientate himself towards the US patent specification mentioned, and in particular the embodiments characterized therein as preferred are also preferred in the context of the present invention. In this context, a section b) which is linear, 7 to 20 amino acids long, and in which each of the amino acids apart from at most one has a guanidino or amidino side chain has proved to be particularly valuable.

US 6,316,003 furthermore discloses a transport polypeptide for intracellular delivery of "freight" molecules with the aid of the HIV tat protein or one or more of its sections. The basic region of the tat protein, by which is understood in particular the section of amino acids 49 to 57, is described therein as being particularly beneficial. For development of the adapter according to the invention, the person skilled in the art will

therefore orientate himself, where appropriate, towards this patent specification. The embodiment examples mentioned therein as preferred are therefore also particularly preferred according to the invention for development of section b) of the adapter according to the invention. These include, in particular, a peptide having the amino acid sequence RKKRRQRRR.

In addition, US 5,888,762 discloses the use of the third helix of a homoeobox peptide for insertion of a macromolecule into a living cell. The person skilled in the art will accordingly use this patent specification if he intends to prepare an adapter according to the invention in accordance with the fourth of the possibilities listed above. In particular, the embodiments described as preferred in US 5,888,762 are also preferred according to the invention. A homoeobox protein fragment with amino acids 43 to 58 of the homoeodomain, in particular from the antennapaedia homoeobox protein, has proved to be particularly advantageous according to the invention; the sections of the homoeobox proteins of engrailed-1, engrailed-2, hoxa-5, hoxc-8 and fushi tarazu corresponding to this are likewise preferred. Section b) of the adapter according to the invention particularly preferably comprises a peptide having the amino acid sequence RQIKIWFQNRRMKWKK, that is to say the amino acid sequence of amino acids 43-58 of the antennapaedia homoeodomain protein of Euprymna scolopes, Accession No. AY 052758.

Finally, US 6,184,038 discloses the use of VP22 of the herpes simplex virus and homologues thereof as a transport protein. The person skilled in the art will accordingly turn to the disclosure of this patent specification if he intends to prepare an adapter according to the invention in accordance with the last of the possibilities listed above. The 34 C-terminal amino acids of the VP22 protein and corresponding analogous and/or homologous peptides, where these mediate the ability to arrange a protein on a cell surface, are particularly preferred according to the invention as section b) of the adapter.

In particular, those adapters in which section b) comprises the basic region of the HIV tat protein and/or a section of the VP22 protein of the herpes simplex virus which mediates anchoring to the cell surface or a part of these peptides can add on to

negatively charged elements of the cell surface of cells of varying origin and are particularly preferred. Addition on to the cell surface of cells of epithelial origin is particularly successful. According to studies to date, addition on to the cell surfaces of cells which tend greatly towards cell adhesion is likewise particularly successful. These particularly preferred adapters according to the invention are therefore advantageously suitable for the preparation of a medicament for combating tumours of epithelial origin and/or for combating tumours having a marked tendency towards cell adhesion. This applies in particular to epithelial carcinomas, sarcomas, glioblastomas and astrocytomas.

It is a particular advantage of the adapters according to the invention, especially those in which section b) comprises the basic region of the HIV tat protein and/or a section of the VP22 protein of the herpes simplex virus which mediates anchoring to the cell surface or a part of these peptides, that they can already achieve a significant improvement in the transduction performance of, for example, an adenovirus for a large number of cell types in relatively low concentrations. The adapter according to the invention is therefore particularly preferably employed with a concentration which is not more than 5,000 times higher than the substance to be coupled, based on the molarity of the linker of the substance to be coupled. For example, if the adapter is to be used for coupling an adenovirus (or correspondingly a substance having a fibre knob protein on its surface), the adapter and adenovirus are mixed, the molar ratio of adapter molecules to fibre knob proteins being not more than 5,000 (adapter) to 1 (fibre knob protein). According to the methods known from the prior art, a considerably higher excess of auxiliary substances is required. Thus, for example, Gratton et al. employ the AntP protein used by them in a ratio of at least 2.5×10^7 (AntP) to 1 (fibre knob) in order to achieve just still detectable minimal effects; good effects are achieved in the method of Gratton et al. only at a ratio of 2.5×10^9 to 1.

The substance to be coupled conventionally has a linker, i.e. a constituent of its surface (such as, for example, a fibre knob protein) which is recognized by section a) of the adapter. The ratio of adapter to linker is then particularly preferably at least 10:1 to 1,500:1, especially preferably 100:1 to 500:1. For example, if an adenovirus is to be arranged on a target cell, in particular for transduction of the target cell, the

concentration of adapter according to the invention (in particular an adapter in which section b) comprises the basic region of the HIV tat protein and/or a section of the VP22 protein of the herpes simplex virus which mediates anchoring to the cell surface or a part of these peptides) is preferably 0.1 to 100 nM, particularly preferably 0.5 to 50 nM, and particularly preferably 1 to 10 nM. Such low concentrations of an adapter according to the invention are already sufficient to achieve a significant improvement in the transduction performance of an adenovirus in a large number of CAR-free cell types.

It has furthermore proved to be beneficial if the adapter according to the invention additionally comprises an oligomerization section for formation of di-, tri- and/or oligomers of the adapter. By providing the oligomerization section, the affinity of the adapter according to the invention for the adenoviral fibre knob protein or the substance to be coupled can be increased permanently in a simple manner. The oligomerization domain of GCN4 protein and/or leucine zipper domains are preferred in particular as the oligomerization section.

The preparation of the adapter according to the invention was particularly successful if this furthermore comprises a leader sequence in order to effect synthesis of the adapter into the rough endoplasmic reticulum, and preferably also into the extracellular space. In this manner, the adapter according to the invention can easily be sluiced out of a eukaryotic cell which produces this. The natural leader sequence of the coxsackie adenovirus receptor is particularly preferred as the leader sequence. The adapter according to the invention expediently has no transmembrane domain of the coxsackie adenovirus receptor. Due to the absence of this transmembrane, the release of the adapter according to the invention from an expressing eukaryotic cell into the extracellular space is additionally simplified.

A nucleic acid having a section which codes for an adapter according to one of the types according to the invention which are described above is furthermore provided according to the invention. Such a nucleic acid simplifies the preparation of the adapter in a host cell or production cell.

The nucleic acid which codes for the adapter can be, in particular, part of a virus genome and can preferably be present therein in the form of an expression unit. A recombinant virus having improved propagation properties in a culture of eukaryotic cells, in particular human and/or animal cells, compared with the wild-type form is accordingly also provided according to the invention. The virus can also have, in particular, improved tumour lysis properties compared with its wild-type form.

In preferred embodiments, the virus for expressing an adapter according to one of the types according to the invention which are described above has, at least in one stage of its multiplication, a virus envelope, and it contains:

- a nucleic acid as just described, and

- a nucleic acid section for expression of a protein of the virus envelope to which section a) of the adapter can couple.

In this context, a virus is understood as meaning a nucleic acid-containing unit, the nucleic acid of which can be multiplied in a host cell, and wherein the nucleic acid can be packed into a particle (virus particle) optionally composed of several units, and wherein the nucleic acid optionally packed into a virus particle can be released from the host cell (for example on the death of the host cell) in order to penetrate into a further host cell. An example of a virus according to the invention is a virus derived from an adenovirus of type 2 and/or 5 and having a nucleic acid for expression of an adapter according to the invention, section a) of which contains a section for recognition of and binding to the adenoviral fibre knob protein.

An adenovirus particle (in this description occasionally also abbreviated to "virus particle") is understood as meaning any body which includes a nucleic acid, or is coupled to it in another manner, which, when it is expressed, effects its own preparation, and has at least one adenoviral fibre knob protein. In particular, the virus particles of conventional adenoviruses are adenovirus particles in the context of the invention. It is furthermore particularly preferable if the adenovirus particle is a virus particles of a virus according to the invention as just described.

The viruses according to the invention render possible in a host cell, in an advantageously simple manner, both preparation of an adapter according to the invention and preparation of a virus envelope or a virus particle which can be recognized and bound by the adapter according to the invention. The virus according to the invention can then propagate in a culture of target cells, without this culture natively having to have particular receptors for coupling the virus to the target cells. As soon as the first target cell is infected and the preparation of the adapter according to the invention has started, this is released from the first target cell and arranged on the cell surface of further target cells. Viruses according to the invention released from the first target cell can then couple via the adapters according to the invention to the further target cells modified in this way and infect these further target cells.

A virus of which multiplication is regulated in a tissue-specific manner is accordingly particularly preferred. The propagation of the viruses according to the invention can be substantially limited to certain tissue (target tissue), for example tumour tissue, in this manner. Preferred promoters which are regulated in a tissue-specific manner and can be used for tissue-specific regulation of the virus multiplication are: promoters for the AFP (alpha foetoprotein) tumour marker, for the prostate-specific antigen (PSA), for the protein subunit of telomerase (hTERT), and promoters which are regulated by transcription factors, in particular by the transcription factors myc, myb, E2F and those transcription factors which are activated and/or expressed more intensively as a result of mitogenic signals.

At the same time or alternatively to this, a virus of which the nucleic acid section which codes for the adapter according to the invention is regulated in a tissue-specific manner is preferred. The expression of the adapter according to the invention can be substantially limited to a target tissue in this manner. The promoters described in the preceding paragraph can in turn be used for tissue-specific regulation.

Those viruses which furthermore comprise a nucleic acid which codes for an agent which induces cell death are likewise preferred, it being particularly preferable if the nucleic acid is equipped such that the agent which induces cell death is expressed in a tissue-specific manner. The corresponding nucleic acid can be, in particular, a section

of the nucleic acid which contains the section which codes for the adapter according to the invention. Preferred agents which induce cell death for which a nucleic acid according to the invention can code are the herpes simplex virus thymidine kinase, and cytosine deaminase. It is preferable in particular if the virus is oncolytic.

If the adapter according to the invention has a higher affinity for the virus particle, in particular for the fibre knob protein, than for the cell surface or for the element(s) of the cell surface which mediate(s) arrangement of the adapter on the cell surface, a virus particle is usually first sheathed with several adapters according to the invention before it is added on to a cell surface.

Furthermore, the use of an adapter according to the invention for non-therapeutic mediation and/or improvement of the coupling of a substance, in particular an adenovirus particle, to a cell surface is a teaching according to the invention. As described above, the adapter according to the invention renders it possible, substantially independently of native receptors or with mediation of widespread cell surface elements of a host cell, to make this receptive for infection with adenovirus particles or for coupling of another substance to be coupled. In this manner, the transformation efficiency, for example, in the infection of a eukaryotic cell culture can advantageously be improved in a simple manner.

The adapters according to the invention are particularly suitable for increasing the infection rate of solid tumours with adenoviruses. The virus titre required for infection can be reduced in this manner, which means that in an animal model or also in humans the hepatotoxicity of a medicament or preparation comprising adenoviruses can be reduced.

A process for non-therapeutic coupling of a substance, in particular an adenovirus particle, to a cell surface is accordingly also provided according to the invention, comprising the steps:

a) exposure to an adapter according to the invention of the substance to be coupled (in particular the adenovirus particle) in order to provide the substance with the adapter, and

b) exposure of the cell surface to the substance which is to be coupled and is provided with the adapter.

In step a), in particular, an adenovirus particle can be provided with the adapter according to the invention. In this context it is advantageous to expose the virus particle to several adapters according to the invention in order to sheathe the virus particle with the adapter according to the invention. In the second step, the target cell is exposed to a dose of the adenovirus particles (at least 1 particle) which have been treated in this way, in order to render possible coupling of at least one particle to the cell surface via the adapter according to the invention. An effective coupling of adenoviruses to target cells which express no or only a small amount of the coxsackie adenovirus receptor on their cell surface and accordingly were hitherto difficult to infect with adenoviruses is rendered possible for the first time in this manner. For example, with the method according to the invention it is possible for the first time to infect stroma fibroblasts with a satisfactory efficiency.

In a therapeutic method, an adapter according to the invention can likewise be employed for mediation and/or improvement of the coupling of a substance, in particular an adenovirus particle, to a cell surface. The latter is advantageous in particular in the context of gene therapy methods with adenoviruses and/or viruses derived therefrom, for example for combating tumours. In a therapeutic method it is likewise preferable first to provide (in particular to sheathe) a virus particle with the adapter according to the invention and then to expose a target cell to the virus particle treated in this way.

It is therefore preferable to use a nucleic acid according to the invention and/or a virus according to the invention for the preparation of a medicament for mediation and/or improvement of the coupling of a substance, in particular an adenovirus particle, to a cell surface, the use of a virus according to the invention being particularly preferred.

The advantages which can be achieved with the therapeutic use just described of the adapter and viruses according to the invention can be realized in a particularly simple manner with such a medicament. In particular, a corresponding medicament for the first time opens up the possibility of also infecting to an extent sufficient for therapeutic purposes cells which hitherto were not infectable, or were infectable only with difficulty, with adenoviruses, in particular tumour cells and stroma fibroblast cells, Fibroblast cells have an essential role for the nutrition of growing tumours, and improving their infectability therefore represents considerable progress in tumour therapy.

A medicament according to the invention for mediation and/or improvement of the coupling of a substance (in particular an adenovirus particle) to a cell surface therefore comprises

- an adapter according to the invention and/or a virus according to the invention and
- a pharmaceutically acceptable carrier.

The medicament can expediently be a preparation consisting of two separate parts, one part of which comprises an adapter according to the invention and the other part of which comprises a virus according to the invention. If the medicament is to be used for mediation and/or improvement of the coupling of an adenovirus particle, it is preferable to mix the two parts of the medicament with one another before administration to a patient, in order to couple the adenoviral fibre knob proteins to the adapter according to the invention; the adapter-loaded viruses formed in this way are particularly suitable for infecting substantially any desired tissue, in particular also that which expresses no or only a small amount of the coxsackie adenovirus receptor.

The processes and uses according to the invention which are described above can be employed in particular in connection with cells of human origin. However, the adapters according to the invention are also suitable for effecting arrangement of a substance, for example an adenoviral fibre knob protein (including a virus particle comprising this protein), on the surface of a target cell of other origin, as long as section b) of the

adapter is chosen such that this can be arranged on the surface of the target cell. A target cell of other origin can be, in particular, a target cell from or in an ape or dog.

The adapters and viruses according to the invention are likewise advantageously suitable for transducing whole tissue and at least in an individual case also whole organisms, in particular animals. They can therefore advantageously be used in gene therapy methods on humans and animals, in particular mouse, rat, dog, sheep, goat, pig, cattle, rabbit, and for the production of a genetically modified animal, in particular mouse, rat, dog, sheep, goat, pig, cattle, rabbit, it being possible for the modification also to include the gonadal pathway.

The invention is explained in more detail in the following with the aid of the examples and the figures, the figures and examples not being intended to limit the subject matter of the invention. The figures show:

Fig. 1 construction diagram of adapters according to the invention;

Fig. 2 photographs of adenovirus-infected cell cultures, wherein the adenovirus has been sheathed with an adapter according to the invention;

Fig. 3 diagram of the mode of action of adapters according to the invention;

Fig. 4 further diagram of the mode of action of adapters according to the invention,

Fig. 5 comparison of the improvement in the transduction performance of various adapters, some of which are according to the invention, for various cell types,

Fig. 6 further comparison of the transduction performance of various adapters, some of which are according to the invention,

Fig. 7 comparison of the transduction performance of various adapters according to the invention with respect to tumour cells of epithelial origin,

Fig. 8 comparison of the transduction performance of various adapters according to the invention with respect to cells of non-epithelial origin,

Fig. 9 results of study to demonstrate the possibility of using conditionally replicating viruses for transduction of appropriate cells,

Fig. 10 results of a study for determination of the permanence of loading of adenoviruses with various adapters according to the invention,

Fig. 11 comparison of the influence of the pH of the culture medium on the transduction efficiency of various adapters, some of which are according to the invention,

Fig. 12 presentation of the influence of administration to various target cells of a substance to be coupled which is delayed with respect to administration of an adapter according to the invention.

Fig. 1 shows a diagram of the construction of four adapter proteins according to the invention in their primary structure. All the adapters have a leader sequence of the coxsackie adenovirus receptor at the N terminus. The leader sequence effects synthesis of the adapter according to the invention into the endoplasmic reticulum of a host cell which expresses the adapter. The leader sequence is followed by the extracellular domain of the coxsackie adenovirus receptor, which corresponds to section a) of the adapter according to the invention. Section b) of the adapter according to the invention follows the extracellular domain in the C-terminal direction. In the four adapters shown (from the top downwards), this is constructed in each particular case as a section of protein VP22 of the herpes simplex virus which mediates anchoring to a cell surface, the basic region of the HIV tat protein, an oligoarginine peptide having 9 arginine radicals and the third helix of a homoeobox protein. A domain which promotes dimerization (here: the leucine zipper from GCN4)

can be arranged between sections a) and b), as shown; it can increase the affinity of the adapter according to the invention for the fibre knob protein.

Fig. 2 shows photographs of cell cultures of NIH3T3 cells which have been infected with adenoviruses using vectors according to the invention. A culture of NIH3T3 cells which was exposed to adenoviruses without a vector according to the invention served as a control (shown top left). The adapters according to the invention were expressed in 293 cells before infection of the NIH3T3 cells and secreted into the cell supernatant. 36 hours after the transfection of the 293 cells with a nucleic acid which codes for the particular adapter according to the invention, the cell supernatant was removed and mixed with LacZ-coding adenoviruses. The adenoviruses provided with a particular adapter according to the invention in this way were added to a culture of NIH3T3 cells in order to infect these. The multiplicity of infection (MOI) was 10. After 48 hours, the cell cultures were investigated for β-galactosidase expression by Xgal blue staining. Cells which were infected with an adenovirus were stained blue here. Infection succeeded best with those adapters according to the invention which had either the basic region of the HIV tat protein (shown top right, "CAR-Tat") or a section of protein VP22 of the herpes simplex virus which mediates anchoring to a cell surface (shown bottom right, "CAR-VP22") in section b). However, an adapter according to the invention with the third helix of the AntP homoeobox protein in section b) also led to a slightly improved infection rate compared with the control (shown bottom left, "CAR-AntP").

Fig. 3 shows a diagram of the mode of action of adapters according to the invention. Adenovirus particles cannot infect CAR-deficient cells or can infect them only poorly (shown top left). The infection rate is increased significantly if the cell to be infected expresses the coxsackie adenovirus receptor on its cell surface (shown bottom left). An adapter according to the invention mediates the adhesion of an adenovirus particle on to a CAR-deficient cell (shown right-hand half). For this, the adapter according to the invention binds with its section a) to a fibre knob protein of the adenovirus particle. Its section b) has a high affinity for the cell surface of the target cell, in particular for the elements of the cell surface which are widespread in the cell type of the target cell. The adapter according to the invention mediates the contact between the cell surface

or the elements of the cell surface for which the adapter has a high affinity and the adenovirus particle. After the contact has been established, the adenovirus particle can be internalized and the infection of the target cell completed.

Fig. 4 shows a diagram of a cell culture partly infected with adenoviruses (cell left outside and right middle) the adenoviruses also coding for an adapter according to the invention. The infected cells both express the adapter according to the invention and release it into the extracellular space. However, they also produce new adenovirus particles; these are released during the cell lysis (shown middle bottom) and are partly sheathed by adapter according to the invention in the extracellular spaces The adenovirus (at least partly) sheathed in this way can infect a further cell (shown left middle, top middle and bottom right), in order to allow preparation there both of the adapter according to the invention and of new adenovirus particles. Infection of the cell culture thus propagates independently.

Fig. 5 shows a comparison of the improvement in the transduction performance of various adapters, some of which are according to the invention, for various cell types. 293 cells were transfected here with expression vectors which code for the particular adapter proteins shown in the part figures. 36 h after transfection of the 293 cells, the supernatant of this cell culture was mixed with adenoviruses (Ad-LacZ) and introduced into the culture medium of the particular cell cultures shown in the part figures. After 4 h the culture medium was exchanged for virus-free culture medium. The cell cultures were incubated under conventional culture conditions for a further 48 h. The transduction performance and infectiousness were determined by β-Gal assay. It can be seen that the transduction performance is improved in all the cell types investigated (RT-101, RKO, BNL, T36274, MCF-7, HT-1080, SAOS-2; SKLU_1) by using the adapters CARex-VP22 and CARex-Tat according to the invention, since the expression of the reporter gene is increased by at least the factor 5 compared with adapter-free infection. In some cell types (SAOS-2, SKLU_1), it was also possible to achieve an improvement in the transduction performance compared with an adapter-free infection by using the adapter CARex-AntP. The other adapters tested were inactive.

Fig. 6 shows a further comparison of the transduction performance of various adapters, some of which are according to the invention. Recombinant, soluble adapter proteins comprising the entire ectodomain of hCAR and basic domains derived from Tat, VP22 and AntP render possible adenoviral infection of CAR-deficient SKLU-1 cells. To determine this finding, 293 cells were transfected with the expression vectors for adapter proteins and control proteins as shown in the legend. Samples of whole cell extracts (top) and corresponding medium supernatants (bottom) of the production cells were analysed by western blotting for adapter expression. As the supernatant analysis shows (Part Figure A), CARex-9xArg is not detectable in the supernatant, in contrast to CARex-Tat or -VP22 and -AntP.

Part Figure B shows the results of a study in which SKLU-1 cells were treated with equivalent volumes of supernatants from adapter-expressing 293 cells (cf. corresponding description to Fig. 5). AdLacZ was added in an MOI of 10 and the infection was carried out for 4 hours. After 48 h, infected cells were detected by X-gal staining. CARex-VP22 and -Tat rendered possible efficient adenoviral infection of this CAR-deficient cell line. In the case of CARex-AntP, only a slight increase was to be observed. All the other control proteins led to no infection of the cells, analogously to the negative control with supernatants of pBluescript-transfected production cells.

Figure 7 shows a comparison of the transduction performance of various adapters according to the invention with respect to tumour cells of epithelial origin. In one study, the results of which are shown in Part Figure A, various target cell types were provided with medium comprising CARex-VP22 or CARex-Tat at a final adapter concentration of 2 nM. AdLacZ (MOI 10) was added immediately and the infection was carried out for 4 h. After further incubation for 48 h, the infection efficiency was estimated by means of X-gal staining. It can be seen that the transduction performance in the case of adapter-assisted infections is on average at least 20 times higher than in the particular adapter-free control.

Part Figure B shows the results of a study in which 1 µg of recombinant CARex-VP22 or CARex-Tat (corresponds to about 4 nM and 8 nM respectively) was diluted in the medium, Ad-GFP (MOI 30) was added and the medium was added to the stated target

cells. Infection was carried out for 4 h. After incubation for 48 h, the fluorescence was observed by microscopy and the infection efficiency was determined by means of FACS analysis. The results show that the adapters mentioned infect non- or weakly permissive cells in a low concentration with up to 100 % efficiency.

Fig. 8 shows a comparison of the transduction performance of various adapters according to the invention with respect to cells of non-epithelial origin. It can be seen that the application of CARex-VP22 or -Tat intensifies adenoviral infection also in cells of non-epithelial origin (e.g. in immune cells and cells from the nervous system).

Part Figure A shows the results of a study in which RAW264.7 macrophages and P388D.1 monocyte macrophages were treated with a medium comprising 2 nM recombinant CARex-VP22 or CARex-Tat as well as AdLacZ (MOI 10, infection time 4 h). The infection efficiency was determined by means of β-Gal assay and X-gal staining. It can be seen that it was possible to increase the transduction efficiency at least 25-fold by using the adapters according to the invention.

In the study leading to Part Figure B, AdGFP (MOI 30) with CARex-VP22 or CARex-Tat (final concentration of 4 and 8 nM respectively) was added to RAW264.7 and p388D.1 target cells. The infection efficiency was determined by fluorescence microscopy and FACS. It can be seen that when adapters according to the invention were used, 88 % and more of the cells of the cell culture were transduced, while in an adapter-free control only 1 % of the cells were transduced.

Part Figure C shows the results of a study in which AdLacZ treated with 2 nM CARex-VP22 or CARex-Tat was added to DC2.4 dendritic cells at an MOI of 10 and 50 respectively for an infection time of 30 min. Quantification was by β-Gal assay and FACS analysis (AdGFP was used in a comparable manner). It can be seen again that when adapters according to the invention were used, it was possible to achieve expression performances which were at least 5 times better compared with a control.

Part Figure D: Immortalized swan cells (ISC) were treated as described in (A). The degree of infection was visualized by X-Gal staining. It can be seen that when

adapters according to the invention were used, it was possible to achieve substantial transfection of the cell culture used.

Fig. 9 shows the results of study to demonstrate the possibility of using conditionally replicating viruses for transduction of appropriate cells. It can be seen from the figure that CARex-VP22- and -Tat-mediated receptor-independent infection intensifies the viral lysis of tumour cells by conditionally replicating and constitutively replicating adenovirus vectors. Tumour cell layers were infected with hTert-Ad (conditionally replicating vector), Ad-wt (constitutively replicating vector) and AdGFP (non-replicating control), an MOI as described in the legend being used. Furthermore, the cells were treated with the non-replicative expression vectors AdCARex-VP22, AdCARex-Tat and AdGFP (control) at an MOI of 25 (valid for weakly permissive cells SAOS-2, HT1080 and MCF-7, as can be seen in A) or at an MOI of 2 (valid for permissive cell lines Huh7 and HepG2, see B). After replication for 6 days, the destruction of the cell layer as a result of viral replication and lysis was visualized by crystal violet staining.

Figure 10 shows the results of a study for determination of the permanence of loading of adenoviruses with various adapters according to the invention. AdGFP was dialysed against DMEM and treated with recombinant CARex-VP22 or CARex-Tat in a 10-fold molar excess corresponding to the available adenovirus fibre knob molecules of AdGFP. Untreated AdGFP served as a control. The virus preparation treated in this manner was loaded at a CsCl density gradient and ultracentrifuged in order to separate off the excess protein. The virus band observed was extracted from the gradient, dialysed against DMEM and quantified by means of OD260 determination. SKLU-1 cells were then infected at an MOI of 50 for 4 h. A high infection rate was to be detected. The results show that CARex-VP22 and -Tat form stable complexes with adenoviral vectors.

Fig. 11 shows a comparison of the influence of the pH of the culture medium on the transduction efficiency of various adapters, some of which are according to the invention. Adapter proteins were prepared as described for Fig. 5 and the pH of the corresponding supernatants were adjusted to a value of from 4.8 to 7.3. The supernatants were then mixed with in each case the same concentrations of Ad-LacZ

and used to infect NIH3T3 cells (30 min exposure of the cells to the virus-containing supernatants, subsequent exchange of the medium for a culture medium). After an incubation time of 48 h, the infection efficiency was investigated by β-galactosidase assay of lysates of whole cells. It can be seen that a higher β-galactosidase expression is achieved at lower pH values. This leads to the conclusion that the adapters according to the invention are also advantageously suitable for improving the transfection of cells within tumour nodules, in which a rather acid medium conventionally prevails.

Figure 12 is a presentation of the influence of administration to various target cells of a substance to be coupled which is delayed with respect to administration of an adapter according to the invention. CARex-VP22 was prepared as described for Fig. 5. The adapter-containing supernatant was added to cultures of target cells (SAOS-2 and SKLU-1). After the time stated on the x-axis, Ad-LacZ was added. The cell cultures were then incubated for 30 min, and the medium was subsequently exchanged for a virus- and adapter-free culture medium as is conventional. After an incubation time of 48 h, the infection efficiency was investigated by β-galactosidase assays of lysates of whole cells. It can be seen that the β-galactosidase expression of SKLU-1 cells is approximately halved with an addition of adenovirus delayed by approx. 30 min, and in the case of SAOS-2 cells this halving occurs only after an addition of adenovirus delayed more than 1.5 h. The CARex-VP22 adapter according to the invention remains available in the culture medium for coupling of the adenovirus to the cell surface for an appropriately long period of time.

Example 1: Preparation of the polynucleotides

The cDNA necessary for generation of a CAR_{1-235}-VP22 fusion protein (an adapter according to the invention) was generated by means of PCR. The cDNA of the human coxsackie virus and adenovirus receptor (hCAR) contained in the plasmid LXSN-hCAR (kindly provided by Dr. DeGregori, Denver, USA) served as the template. The oligonucleotide 5'-GT*GGTACC* **ATG** GCG CTC CTG CTG TGC TTC GTG C-3' (Kpn I cleavage site in italics, natural start codon of hCAR in bold) was used as the 5' primer and the oligonucleotide 5'-TA*GCGGCCGC***C TTT ATT TGA AGG AGG GAC AAC**

GTT TAG ACG C-3' (Not I cleavage site in italics, complementary to bases 776 to 807 of hCAR (Entrez Pubmet Accession No.: NM 001338) in bold) was used as the 3' primer. The fragment formed contains the leader sequence of hCAR and the extracellular domain thereof. In the PCR, 5 cycles were carried out at 49 °C and 30 cycles at 55 °C, using Pfu polymerase. The PCR product was purified with the aid of an agarose gel electrophoresis and the ends were regenerated by digestion with Kpn 1 and Not 1. This fragment was then inserted into the corresponding restriction cleavage sites of the vector pVP22mycHis 2 (Invitrogen), resulting in the plasmid pCAR(ex)-VP22. The plasmids for fusion proteins of the extracellular domain of hCAR with the cell adhesion domains of HIV-TAT, AntP and a 9 x arginine sequence (9xArg) were cloned as follows:

For generation of pCAR(ex)-TAT_{48-57} the oligonucleotide 5' AAA*GC GGC CGC* GGA GGA GGA AGT GGA GGA GGA GGA **GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA** GG*T CTA GA*AA-3' (sense oligonucleotide, bold corresponds to bases 5518-5547 of the HIV genome, Accession No.: NC 001802, coding for amino acids 48-57 (sequence: GRKKRRQRRR) of the TAT protein, cleavage sites for Not I and Xba I in italics) and a correspondingly complementary antisense oligonucleotide (5'-TTTCTAGACCTCTTCGTCGCTGTCTCCGCTTCTTCCTGCCTCCTCCTCCTCCACTT CCTCCTCCGCGGCCGCTTT-3') were hybridized, digested with Not I and Xba I and inserted into the corresponding cleavage sites of the plasmid pCAR(ex)-VP22. This was cleaved beforehand with the restriction enzymes Not 1 and Xba 1 and the cDNA which codes for VP22 was separated off by agarose gel electrophoresis.

The AntP and 9xArg variants were generated by comparable working steps.

The oligonucleotide 5'AAA*GC GGC CGC* GGA GGA GGA AGT GGA GGA GGA GGA **CGT CGC CGA CGG AGA AGG AGA CGT AGA** GG*T CTA GA*AA-3' (sense oligonucleotide, bold corresponds to the DNA which codes for the amino acid sequence RRRRRRRRR, cleavage sites for Not I and Xba I in italics) and a correspondingly complementary antisense oligonucleotide (5'-TTTCTAGACCTCTACGTCTCCTTCTCCGTCGGCGACGTCCTCCTCCTCCACTT

CCTCCTCCGCGGCCGCTTT-3') were used as DNA which codes for 9xArg. Name of the resulting plasmid: pCAR(ex)-9xArg.

The oligonucleotide 5'AA*GC GGC CGC* GGA GGA GGA GGA **AGA CAG ATC AAA ATA TGG TTC CAA AAC CGG CGC ATG AAA TGG AAG AAA** GG*T CTA GA*AA-3' (sense oligonucleotide, bold corresponds to bases 184-231 of the partial cds for Euprymna scolopes antennapaedia homoeodomain protein, Accession No.: AY052758, coding for amino acids 62-77, sequence: RQIKIWFQNRRMKWKK, cleavage sites for Not I and Xba I in italics) and a correspondingly complementary antisense oligonucleotide (5'-TTTCTAGACCTTTCTTCCATTTCATGCGCCGGTTTTGGAACCATATTTTGATCT GTCTTCCTCCTCCTCCGCGGCCGCTT-3') were used as the DNA coding for AntP. Name of the resulting plasmid: pCAR(ex)-AntP_{62-77}.

All the oligonucleotides used also have several glycine/serine radicals in the 5' direction of the cell adhesion domain in order to separate this domain spatially from the CAR domain.

The abovementioned plasmids were sequenced and prepared with the Qiagen Endofree Plasmid Maxi Prep Kit. Production of analytical amounts of CAR fusion proteins was carried out by transfection of 293 cells using the calcium precipitation method or liposome-forming agents. 24-48 h after the transfection, it was possible for the fusion proteins contained in the cell culture supernatants to be used further for analytical purposes.

Example 2: Preparation of the adenoviruses which code for CAR(ex) fusion proteins for preparative protein production from infected cell cultures

The adenovirus which contains an expression unit for CAR_{1-235}-VP22 was generated with the aid of the cloning system according to Mizuguchi and Kay (Hum. Gene Ther. 1998, *9*(17): 2577-83). A modified variant of the vector pHM3 (pHM3/pBK-CMV) which contains, in the MCS of pHM3, the complete expression cassette (CMV-promoter/MCS/SV40-polyadenylation signal) of the vector pBK-CMV (Stratagene) was

used as the starting point for the shuttle vector. The cDNA for CAR_{1-235}-VP22 was prepared from the plasmid pCAR(ex)-VP22 by Hind III digestion, treatment with Klenow fragment and subsequent Pme I digestion and inserted into the Sma I cleavage site of the vector pHM3/pBK-CMV. Correct orientation of the resulting construct (p4622) was checked by Kpn I digestion. Cloning of the adenoviral plasmid was carried out by isolation of the PI-Sce/I-Ceu fragment from p4622 and ligation thereof into the corresponding cleavage sites of the adenoviral vector pAdHM4. The ligation resulted in the construct pAd4679. 293 cells were transfected with a Pac I-linearized preparation of this vector in order to generate infectious particles. After occurrence of a cytopathic effect, the cells were harvested and the infectious particles were released by freezing and thawing three times. The cell debris was centrifuged off and the supernatant was employed for the preparation of virus preparations of higher titre (designation of the adenovirus obtained: Ad4679). Obtaining of higher titres of recombinant viruses was likewise carried out in 293 cells and subsequent purification of virus-containing lysates via CsCl gradient ultracentrifugation.

Generation of the adenovirus with an expression cassette for CAR_{1-235}-TAT_{48-57} was carried out with the aid of the recombination system of He and Vogelstein (He et al., Proc. Natl. Acad. Sci. USA 1998, *95*: 2509-2514). The cDNA which codes for CAR_{1-235}-TAT_{48-57} was isolated from the plasmid pCAR(ex)-TAT by Hind III and Pme I digestion and ligated into the Hind III and EcoR V cleavage sites of the vector pShuttle-CMV. The ligation resulted in the construct p4923. The construct p4923 was linearized with Pme I and then mixed with the adenoviral vector AdEasy 1. BJ5183 E. coli capable of recombination were electroporated with this batch. The recombinant adenovirus plasmid thereby generated was called p5091. 293 cells were transfected with a Pac I-digested preparation of this vector in order to generate infectious particles. After occurrence of a cytopathic effect, the cells were harvested and the infectious particles were released by freezing and thawing three times. The cell debris was centrifuged off and the supernatant was employed for the preparation of virus preparations of higher titre (designation of the adenovirus obtained: Ad5091).

Example 3: Preparation of the recombinant CAR fusion proteins from the supernatants of adenovirally infected cells

The recombinant CAR(ex) fusion proteins have a 6 x histidine motif on their C terminus which is capable of complexing positively charged Ni ions. The fusion proteins can be purified from complex protein mixtures in this manner with the aid of nickel ions bound to a solid phase.

The adenoviruses Ad4679 and Ad5091 described previously were used as vectors for production of CAR fusion proteins in infected host cells.

All adherent, adenovirally infectable cell types, such as e.g. COS-1, HepG2, Huh 7 etc., are suitable in principle as cell lines for production of the recombinant proteins. Infected COS-1 cells gave high amounts of active CAR fusion proteins in the supernatant and were therefore preferably employed. COS-1 cells were infected at a confluence of 80-90 % with Ad4679 or Ad5091 with an MOI of 10-50 in DMEM (supplemented with 2 % foetal calf serum) and incubated for 48 h at 37 °C and 5 % CO_2. After 48 h, 1/10 vol. of 10x application buffer (500 mM NaH_2PO_4, 3 M NaCl, 100 mM imidazole) were added to the infected cell cultures and incubation was carried out in the incubating cabinet for a further 1 min. Thereafter, the cell culture supernatant was removed for the subsequent purification by column chromatography (medium was added to the infected cells for a further incubation period, and incubation was carried out again for 48 h). The supernatants obtained were collected, and any entrained cells were separated off by centrifugation at 1,000 x g for 10 minutes and sterile filtration with a 0.22 µm filter. Thereafter, the column chromatography was carried out. For this, Ni-NTA agarose (Qiagen) was equilibrated with application buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) and the cell culture supernatants described above were applied. The column was then washed with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole). Elution of the CAR fusion proteins was carried out with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM L-histidine). The concentration and function of the CAR fusion proteins in the eluate was determined by BioRad protein assay, SDS gel electrophoresis with Sypro orange staining and

infection experiments. Sufficiently concentrated eluates were dialysed against 25 % glycerol in DMEM medium, shock-frozen in liquid nitrogen and stored at 80 °C.

The determination of the ratio of the adapter protein to be used to the amount of virus-associated fibre knob is described in the following.

The virus concentration is determined with the usual titration methods. This is carried out e.g. spectrophotometrically by measuring the OD_{260} for determination of the total concentration of viral particles, 1 OD corresponding to 1 x 10^{12} adenovirus particles/ml. The infectious particle concentration can be determined by plaque assay, rapid titre assay (BD Biosciences) or by limiting dilution and subsequent statistical calculation.

The adapter protein concentration in affinity chromatography preparations is determined by conventional protein determination methods (BioRad Protein Microassay, Lowry et al., OD230/260) taking into account a molecular weight for CARex-VP22 of approx. 68 kD and for CARex-Tat of approx. 38 kD. For simplification, the following calculations are based on the assumption of 100 % intact fibre knob protein in the adenovirus.

Approx. 250 ng CAR-Tat or 500 ng CAR-VP22 (in the following batch corresponding to a concentration of approx. 2 nM) are sufficient for mediation of a virtually 100 % infection of 1 x 10^6 non-permissive target cells (e.g. SKLU-1 or NIH3T3) with Ad-LacZ at an MOI (multiplicity of infection) of 25 in an infection volume of 3 ml.

At an MOI of 25, this corresponds to a number of 2.5 x 10^7 infectious particles. Since usually in AdLacZ preparations approx. every tenth particle is infectious, this corresponds to a total particle count of 2.5 x 10^8. Since adenoviral particles each have 12 fibre knob molecules, this corresponds to a fibre knob concentration of 1 x 10^9/ml or 1 x 10^{12}/l. The adapter protein is employed at a concentration of 2 nM, i.e. with 1.2 x 10^{15}/l. A recommended molar ratio of adapter protein (assuming 100 % functional protein) to the theoretical number of fibre knobs is therefore, in particular, 1,200. The molar excess of 1,200 serves to compensate for factors such as dissociation equilibrium, adapter uptake by the target cells and the content of non-functional

adapter protein. At lower preincubation volumes or infection volumes, it is to be expected that a lower excess will also be sufficient, such as e.g. 100-150. In the case of in vivo infections also (mouse, intravenous injection into the tail vein), this adapter/fibre knob ratio is sufficient with preincubation of the required amount of virus (e.g. 1 x 10^{10}/20 g of mouse) with the calculated amount of adapter in an injection volume of 300 µl. The amount of non-bound adapter can be separated off beforehand by a density gradient centrifugation (CsCl gradient).

Gratton mentions a concentration range of from 50 µM to 5 mM in 100 µl of solution volume with 1 x 10^6 infectious particles of the adenovirus to be coupled in order to achieve a significant improvement in transduction.

50 µM correspond here to an absolute use of AntP of 5 nmol, corresponding to an amount of 3 x 10^{15} AntP molecules. The number of linker molecules (fibre knob) here is 1.2 x 10^8, given the assumption that every 10th particle of the virus employed is in fact infectious, and an adenovirus particle has 12 fibre knob molecules. The calculated ratio of adapter (AntP) to linker (fibre knob) is accordingly 3 x 10^{15} / 1.2 x 10^8 = 2.5 x 10^7, or calculated analogously for the total preferred range of use according to Gratton of 50 µM – 5 mM: 2.5 x 10^7 – 2.5 x 10^9.